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(54) Title: METHOD OF USING PRESERVED CONTROL CELLS IN THE CALIBRATION OF FLUORESCENT AND LIGHT SCATTER MEASUREMENTS

(57) Abstract

The invention describes a method of using differently labelled, reconstituted preserved cells as control cells in multiple color assays. These cells are used to calibrate light scatter and fluorescent intensity measurements. The use of said control cells overcomes the preservation and calibration difficulties experienced in the conventional use of fresh cells and polymer beads as controls in determining the compensation adjustments required in multiple color flow cytometry assays as a result of the overlapping wavelength regions encountered.

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METHOD OF USING PRESERVED CONTROL CELLS IN THE CALIBRATION OF FLUORESCENT AND LIGHT SCATTER MEASUREMENTS

TECHNICAL FIELD

The invention relates to the use of labelled preserved cells as controls to make compensation adjustments for the overlapping wavelength emissions of multiple fluorescent substances used as labels in multiple color biological analyses by flow cytometry. The invention also describes the preparation of preserved labelled cells which have stability and shelf life characteristics not previously realized.

BACKGROUND ART

Control cells are essential for the accuracy and precision of clinical tests and immunoassays. In assays which use light scatter and fluorescent labels to identify cells and/or cellular components, control cells are used to form the basis for making compensation adjustments for areas of fluorescent overlap. The use of control cells and the making of the compensation adjustments is necessary to insure the reliability and accuracy of the test equipment and methods, and to insure reproducibility through time and from laboratory-to-laboratory.

In assays using light scatter and fluorescent labels, cells and/or cellular substances are determined on the basis of either direct interaction with the fluorescent substance or indirect interaction via bonding of the fluorescent substance to a biological substance, for example, a monoclonal antibody, plant lectin and other substances which may be involved in the reaction with the cells or cellular components. When only a single fluorescent substance is used in an assay, any compensation adjustments are not difficult to do. However, such adjustments become very important and are more difficult when multiple fluorescent labels are utilized in multiple color analysis. The problem arises

because many of the labels used in these analyses have overlapping emission wavelength regions. The fluorescent light produced in the overlap region is the sum of the light produced by overlapping wavelengths of the individual fluorescent substances. The problem is particularly important in flow cytometry where the objective is to label cells with at least two distinct fluorochromes which reflect discrete cellular components by detecting a signal proportional to each fluorochrome and unbiased by any contribution from the other fluorochrome. Unless adjustments are made for the overlapping wavelengths, false "positive" readings may occur. It is not possible to choose wavelength filters which will optimally transmit the light of one overlapping] fluorochrome and totally block the light of the other fluorochrome, thereby avoiding the false positive readings. Most multiple color analytical systems provide "compensation" to eliminate the false positive readings.

Compensation, as used herein, is an electronic means of computing and subtracting overlapping fluorescent signals, i.e., the overlap contribution from a second fluorochrome is subtracted from the fluorescence reading of a first fluorescent label on the cells being analyzed. When cells bearing the second fluorochrome are analyzed, the process is reversed and the overlap contribution from the first fluorochrome is subtracted from the fluorescence reading of the second fluorescent label. In order to determine the proper amount of electronic compensation, there must be some method of determining whether too much or too little signal is subtracted relative to the specific fluorochrome and the targeted cells or cellular components.

Traditionally, fluorescently labelled fresh normal blood samples or latex beads have been used as controls in light scatter analyses to determine the necessary adjustments and the appropriate amount of electronic compensation. However, fresh normal blood samples are

not entirely satisfactory because they can be used as a compensation controls for only a short period of time due to the degradation which such samples undergo during storage. New normal blood samples must be obtained continuously. As a result, fresh blood samples are neither amenable to commercialization because of their lack of stability nor do they provide a uniform baseline for the comparison of samples separated in time. Latex beads offer better stability and a uniform baseline, but their light scatter characteristics are different from the light scatter characteristics of biological cells or cellular components. Consequently, the use of latex beads provides compensation values which are different from those values obtained using real cells.

Another consideration in flow cytometry is that is important that the size, shape and structure of the labelled calibration substance have a direct relationship to the size, shape and structure of the cells or cellular components being analyzed. For example, a large labelled spherical calibration or control particle would not be a proper match for an unknown consisting of small rectangular particles. For this reason, biological cells are preferred over latex beads as controls.

This invention provides a method for using preserved, labelled biological cells as control cells for light scatter measurements and fluorescent calibration. A provision providing for such cells enables the analyst to select the proper cells for making compensation adjustments and provides the ability to establish long term baselines for the comparison of samples analyzed at different points in time.

DISCLOSURE OF INVENTION

The invention describes a method of using labelled reconstituted preserved cells as control cells to calibrate light scatter and fluorescent intensity measurements in multiple color assays using two or more fluorescent labels and to provide for compensation adjustments in such assays which eliminate false positive

readings caused by the overlapping wavelengths of different fluor scent substances. The compensation factors used in the adjustments are determined electronically by the analytical instrument. The compensation factors prevent the appearance of false positive readings during the assay.

The assays with which the method is used are biological cells assays. The method is an improvement over conventional techniques which require fresh cells or which use polymeric beads. The use of labelled preserved cells according to the invention enables the analyst to establish a long term baseline for the comparison of samples assayed at different points in time. The invention also describes preferred labelled preserved cells for use as the control cells and a method of preparing the same.

BRIEF DESCRIPTION OF DRAWINGS

FIG 1A illustrates the gating, with no compensation adjustments, of the preserved cells on the basis of forward light scatter (FS) and log side light scatter.

FIG. 1B illustrates false positive fluorescent staining of cells in Quad 2 due to lack of fluorescent compensation adjustments.

FIG. 1C further illustrates false positive fluorescent staining in Quad 2 due to lack of compensation adjustments.

FIG. 1D illustrates that even without compensation adjustments there is no overlap region for FITC and ECD fluorescent emissions.

FIG. 2A illustrates the gating, with compensation adjustments of the preserved cells on the basis of forward light scatter and log side light scatter.

FIG. 2B illustrates the correct fluorescent reading obtained after sitting the appropriate compensation for overlapping fluorescent emissions.

FIG. 2C illustrates that with the appropriate fluor scent compensation for overlapping emission wavelengths, there are no false positive r adings in

Quads 1 and 2.

FIG. 2D further demonstrates that with the appropriate fluorescent compensation, these are no false positive readings in Quads 1 and 2.

BEST MODE FOR CARRYING OUT THE INVENTION

The invention describes a method which uses preserved cells to determine light scatter and compensation adjustments for wavelength emissions of multiple fluorescent substances used as labels in multiple color biological analysis. In general, any preserved, labelled cells which retain their authentic structural and antigenicity characteristics can be used according to the invention. For example, one may practice the invention by using lyophilized labelled cells as control cells. Lyophilized cells may be prepared according to U.S. Patent No. 5,059,518 issued October 29, 1991 for STABILIZED LYOPHILIZED MAMMALIAN CELL AND METHOD OF MAKING SAME. The teachings of Patent No. 5,059,518 are incorporated herein by reference. Other methods of preserving cells, bacteria, and other biological substance may be found in U.S. Patent Nos. 3,261,761, 4,874,690, 4,206,200 and 4,246,349; European Patent Application No. 0 259 736, published March 16, 1988; and International Patent Applications published under the Patent Cooperation Treaty as Nos. WO 86/03938 (published July 17, 1986), WO 87/00196 (published January 17, 1987) and WO 89/06976 (published August 10, 1990); and Japanese Patent Application Nos. 57-7419, 56-12317 and 58-131913. The selection criteria for any preserved cells used according to the invention is that when such cells are reconstituted, washed or other prepared for use, they retain their authentic structural and antigenic characteristics, and that these characteristics are the same as corresponding fresh cells. Such preserved cells, after reconstitution, washing or otherwise being prepared for use in the claimed invention are hereby defined as "reconstituted control cells" or simply "control cells."

The labels used in the invention are fluorescent

substanc s. The labelling may be done by direct reaction between th label and biological cells or cellular components. Alternatively, the label may be attached to (1) a reactive biological material, such as, a monoclonal antibody or (2) a reactive non-biological organic substance, for example, a carboxylic acid chloride, prior to attachment to the cells or cellular components. For example, fluorescent labels may be attached to a selective reactant substance such as a monoclonal antibodies and the labelled monoclonal antibodies then conjugated to cells. The preferred method is to attach the label to a selectively reactive substance, and most preferably, to a monoclonal antibody.

The fluorescent labels may be selected from fluoroscein, fluoroscein isothiocyanate (FITC), rhodamine, tetramethylenerhodamine isothiocyanate (TRITC), sulforhodamine 101 acid chloride (Texas Red), phycoerythrin (PE), allophycocyanin, phycoerythrin-Texas Red (PETR), 4-methylumbelliferone and other fluorescent substances known or found useful in the analysis of biological substances. The conjugation of the label to the monoclonal antibody can be performed by any suitable and known method including direct reaction between the monoclonal antibody and the label or the use of bridging groups to connect the monoclonal antibody and the label.

The labelled lyophilized cells used herein as control cells may be prepared by reconstituting cells lyophilized according to Patent No. 5,059,518 and incubating the reconstituted cells with either a polyclonal or monoclonal antibody. Monoclonal antibodies specific to an antigenic site present on the cells are preferred. The antibody may be labelled before incubation with the reconstituted cells or it may be labelled after it has been attached to the reconstituted cells. The former is preferred. The resulting labelled cells are then used according to the claimed invention.

Alternatively, fresh cells may be labelled, for

example, by incubating the fresh cells with an antibody. The antibody may b labelled before or after incubation. Again, the former is preferred. After labelling, the cells are lyophilized according to said Patent No. 5,059,518. It has been determined that preservation by the method of said patent does not destroy or decrease the fluorochrome's fluorescent ability as is known to occur using other methods of preservation. Prior to their use as control cells according to the herein invention, the lyophilized labelled cells are reconstituted.

It is also within the scope of the invention to use cells which have been labelled directly. Directly labelled cells are those in which the label is attached to the cell without the use of an intervening antibody or other substance.

Example Of Practicing The Invention.

An anti-CD8 monoclonal was labelled with phycoerythrin (RD1, obtainable from Coulter Corporation of Miami, Florida) to produce a labelled antibody designated as LFL2. An anti-CD4 monoclonal antibody was labelled with fluoroscein isothiocyanate (FITC) to produce a species designated as LFL1. Lastly, a negative control antibody (IgG_2b) was labelled with ECD (energy coupled dye, Coulter Corporation, Miami, Florida) to produce a species designated LFL3. (All antibodies are available from Coulter Corporation, Miami, Florida). These three labelled antibody species were used to label lymphocytes preserved by the method described in patent No. 5,059,518. Tricolor (RD1, FITC, EDC) flow cytometric analysis of the cells using this plurality of labels was then performed using no compensation as illustrated in Fig. 1 and using compensation as illustrated in Fig. 2. As used herein the use of a "plurality" of fluorescent labels signifies the use of two or more labels. The maximum number is dependent solely on th capabilities of the instrument used in the assay, i.e. it may be two, three, four or more labels.

FIG. 1A illustrates the gating of the pr s rved cells on the basis of forward light scatter (FS) and log side light scatter (LSS) included in electronic gate (1).

FIG. 1B (LFL2/LFL1) illustrates the false positive fluorescent staining of cells in Quadrant (Quad) 2 due to the lack of fluorescence compensation, such that, there appears CD8-FITC positive cells in Quadrant 1 (LFL2+); CD4-RD1 positive cells in Quadrant 4 (LFL1+); LFL1(-) LFL2(-) cells in Quadrant 3; and LFL1(+) LFL2(+) cells in Quadrant 2. The dual positive cells seen in Quadrant 2 are the result of the FITC emission wavelength overlapping into LFL2 (overlap between FITC and RD1 emissions). It should be noted that the mean channel of LFL2 in Quad 3 (x-axis = 0.146) is quite different from LFL2 in Quad 1 (x-axis = 0.233) and that LFL1 in Quad 3 (y-axis = 0.128) is quite different from LFL1 in Quad 4 (y-axis = 0.222).

FIG. 1C (LFL3/LFL2) illustrates the false positive fluorescence is due to the fact that there is no fluorescence compensation for overlapping wavelengths (refer to FIG. 1B). The dual positive cells seen in Quadrant 2 are the result of the emission wavelength from LFL2 (RD1 emission) overlapping with LFL3 (ECD emission).

FIG. 1D (LFL3/LFL1) illustrates that without compensation, the separate emissions of FITC and EDC do not overlap. Consequently, there is no indication of dual positive cells.

FIG. 2 results were obtained is the same manner as realized for FIG. 1, except that there was compensation for overlapping wavelengths. FIG. 2A illustrates the gating of the preserved cells on the basis of forward light scatter (FS) and log side light scatter (LSS) included in electronic gate (1).

FIG. 2B (LFL2/LFL1) illustrates the correct fluorescent reading which is obtained after setting the appropriate compensation for overlapping fluorescent emissions using antigenically preserved cells as control cells. It should be noted that the mean channel of LLF2

in Quad 3 (x-axis = 0.169) LFL2 in Quad 1 (x-axis = 0.150) are similar. Likewise, the mean channel of LFL1 in Quad 3 (y-axis = 0.147) is similar to the mean channel of LFL1 in Quad 4 (y-axis = 0.143). In contrast to FIG. 1B, there are no false positive readings.

FIG. 2C (LFL3/LFL2) illustrates that when there is appropriate fluorescence compensation, no LFL3 positive cells are indicated in Quads 1 and 2.

FIG. 2D (LFL3/LFL1) illustrates that with appropriate fluorescence compensation, no LFL3 positive cells are indicated in Quads 1 and 2.

As seen in FIGS. 2A-2D, preserved cells can be used to adjust the instrument's light scatter to incorporate the preserved cell population of interest, for example, lymphocytes and sub-populations thereof. To verify that the color compensation is appropriate for a given signal, the mean channel of the positive population and the mean channel of the negative population should be equal or nearly equal. This means that for the phycoerythrin-RD1 fluorescence, the mean channel of the fluorescence of the cells labelled with FITC should be equivalent or nearly equivalent to the mean channel of the negative cells labelled with phycoerythrin RD1. For the FITC fluorescence, the mean channel of the fluorescence of the cells labelled with phycoerythrin-RD1 should be equivalent or nearly equivalent to the mean channel of the negative cells labelled with FITC. When the analytical instrument is so adjusted, the correct overlapping wavelength compensation is achieved and used in the analysis.

The procedure can be used with two or more fluorescent labels. The use of antigenically preserved cells, as shown in the Examples, enables the compensation factor to be reproducible over time and between different instruments and different laboratories.

We claim:

CLAIMS

1. A method of calibrating a flow cytometer instrument for use in a predetermined multiple color assay of at least a pair of different biological cells which have been labelled with fluorescent dyes having different, but overlapping excitation wavelength regions, said method comprising, preparing at least a pair of control cells selected to be suitable for conducting the said multiple color assay, each member of the pair cells having a different fluorescent label conjugated thereto, said labelled control cells having different, but overlapping excitation wavelength regions and comprising reconstituted preserved cells having the desired biological characteristics for determining said measurements when processed through said flow cytometer, processing said labelled control cells through said flow cytometer to ascertain relevant measurements which denote the overlapping excitation wavelength region, said relevant measurements thereafter determining the required compensation adjustments to be used in conducting the assay of the selected biological cells with said instrument.

2. A method of calibrating a flow cytometer for use in a predetermined multiple color assay of a sample containing at least two different types of biological cells which have been labelled with fluorescent dyes that have different, but partially overlapping emission wavelength regions, said methods comprising:

(a) preparing a test sample of at least two sets of control cells selected as suitable for conducting said multiple color assay, each set of cells having only one of said fluorescent dyes conjugated thereto, said control cells being from the group consisting of reconstituted preserved cells having the required biological characteristics necessary for performing said predetermined assay and labelled with said fluorescent dyes either b fore preservation or after reconstitution;

(b) proc ssing said labelled control cell sets sequentially through said cytometer to determine the relevant intensity measurements for each dye throughout its emission spectrum; and

(c) making the necessary compensation adjustments to said cytometer in order to obtain an accurate assay within the overlapping region of the emission spectra of said dyes;

wherein when said method is completed, said flow cytometer is calibrated to accurately assay cells within a sample that are labelled with said fluorescent dyes.

3. The method of claim 2 wherein said control cells are selected from the group consisting of:

(a) cells in which the dyes are attached directly to said cells and

(b) cells in which the dyes are attached to said cells by mean of a bridging group between said dye and said cells.

4. The method of claim 3 wherein said bridging group is an antibody.

5. The method of claim 3 wherein a single type of cells is classified into subsets by labelling said single type of cells with at least two selected fluorescently labelled monoclonal antibodies.

6. The method of claim 3 wherein said control cells are labelled, reconstituted lyophilized cells.

7. The method of claim 1 in which at least an additional dye-labelled control cell is prepared for use in the assay which does not overlap in the spectrum of the pair of dye-labelled control cells at the excitation wave length used to excite said pair of dye-labelled control cells.

8. The method of claim 1 in which said control cells are labelled, reconstituted lyophilized cells.

9. Th method of claim 8 wherein said control cells are s lected from the group consisting of:

(a) cells in which the dyes are attached directly to said cells and

(b) cells in which the dyes are attached to said cells by mean of a bridging group between said dye and said cells.

10. The method of claim 9 wherein said bridging group comprises an antibody.

11. The method of claim 1 in which at least an additional dye-labelled control cell prepared for use in the assay which does overlap in the spectrum of only one of the pair of dye-labelled control cells at the excitation wavelength used to excite said pair of dye-labelled control cells.

AMENDED CLAIMS

[received by the International Bureau on 25 May 1994 (25.05.94); original claims 1,2,5,7 and 11 amended; remaining claims unchanged (3 pages)]

1. A method of calibrating a flow cytometer instrument for use in a predetermined multiple color assay of a sample containing at least two types of cells, each type of which has been labelled with a different fluorescent dye, said different dyes having overlapping excitation wavelength regions, said method comprising:

(a) preparing as control cells at least two types of biological cells selected to be suitable for conducting said multiple color assay, each of said type of cells having a different fluorescent label conjugated thereto, said labelled control cells having different but overlapping excitation wavelength regions and comprising reconstituted preserved cells having the desired biological characteristics for said assay and for measuring said overlapping wavelength regions when said cells are processed through a flow cytometer;

(b) processing said labelled control cells through said flow cytometer to ascertain relevant measurements which denote the overlapping excitation wavelength region;

(c) using said relevant measurements to determine the required compensation adjustments to be used in conducting the assay of the selected biological cells with said flow cytometer; and

(d) making said compensation adjustments.

2. A method of calibrating a flow cytometer for use in a predetermined multiple color assay of a sample containing at least two different types of biological cells which have been labelled with fluorescent dyes that have different, but partially overlapping emission wavelength regions, said method comprising:

(a) preparing test samples containing at least two types of control cells selected as suitable for conducting said multiple color assay, and within a given t st sample only one type of cells has only one of said fluorescent dyes conjugated th reto, said control cells

consisting of reconstituted preserved cells having the required biological characteristics necessary for performing said predetermined assay and labelled with said fluorescent dye either before preservation or after reconstitution;

(b) processing said labelled control cell samples sequentially through said cytometer to determine the relevant intensity measurements for each cell-conjugated dye throughout its emission spectrum; and

(c) making the necessary compensation adjustments to said cytometer using the measurements of step (b) in order to obtain an accurate assay within the overlapping region of the emission spectra of said dyes;

wherein when said method is completed, said flow cytometer is calibrated to accurately assay cells within a sample in which the same type of cells are labelled with said fluorescent dyes.

3. The method of claim 2 wherein said control cells are selected from the group consisting of:

(a) cells in which the dyes are attached directly to said cells and

(b) cells in which the dyes are attached to said cells by means of a bridging group between said dye and said cells.

4. The method of claim 3 wherein said bridging group is an antibody.

5. The method of claim 3 wherein a single type of cells is classified into subsets by labelling said type of cells with at least two selected fluorescently labelled monoclonal antibodies, each of said fluorescent labels being on a different antibody.

6. The method of claim 3 wherein said control cells are labelled, reconstituted lyophilized cells.

7. The method of claim 1 in which at least an additional dye-labelled control cell is prepared for use in th assay which does not overlap in th spectrum of the two dye-labelled control cells at the excitation wave length used to excite said two dye-labelled control

cells.

8. The method of claim 1 in which said control cells are labelled, reconstituted lyophilized cells.

9. The method of claim 8 wherein said control cells are selected from the group consisting of:

(a) cells in which the dyes are attached directly to said cells and

(b) cells in which the dyes are attached to said cells by means of a bridging group between said dye and said cells.

10. The method of claim 9 wherein said bridging group comprises an antibody.

11. The method of claim 1 in which at least an additional dye-labelled control cell prepared for use in the assay which does overlap in the spectrum of only one of the two of dye-labelled control cells at the excitation wavelength used to excite said two dye-labelled control cells.

Fig. 1a

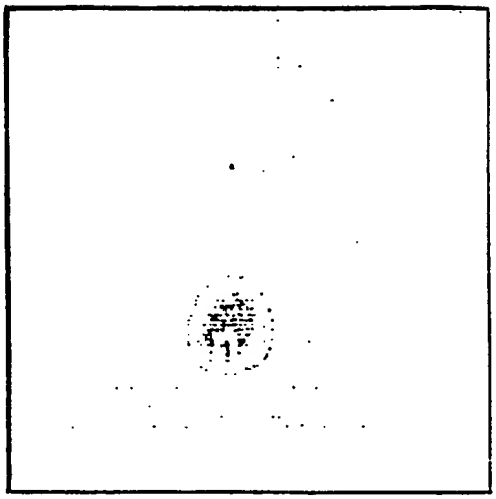


Fig. 1b

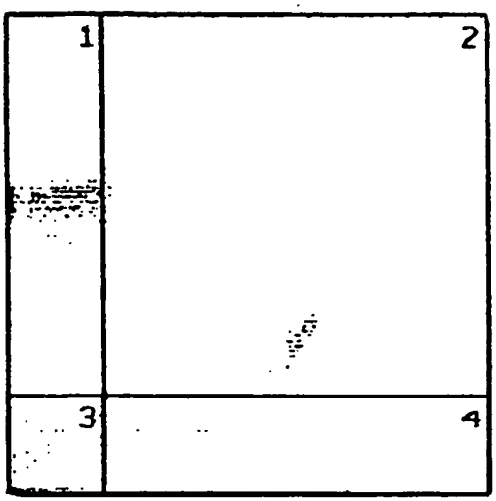


Fig. 1c

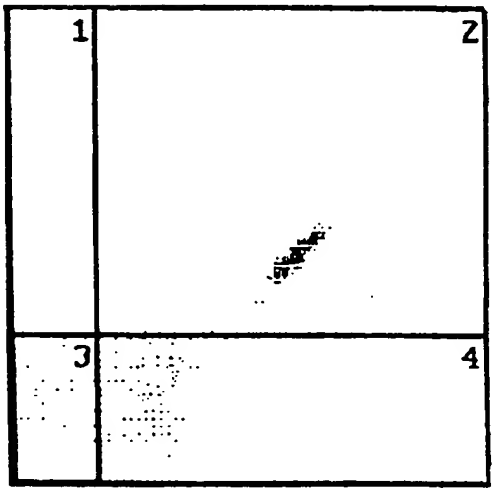


Fig. 1d

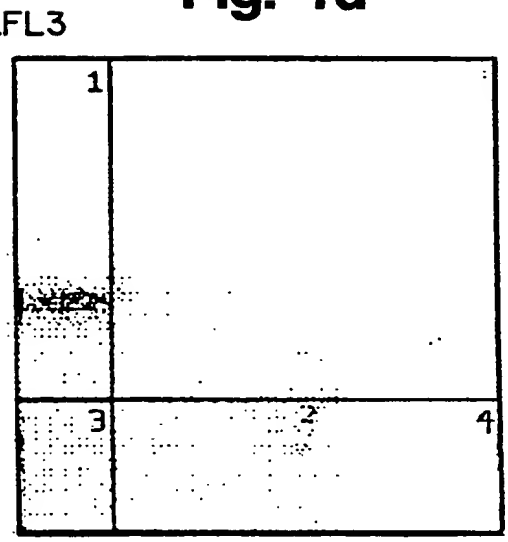


Fig. 2a

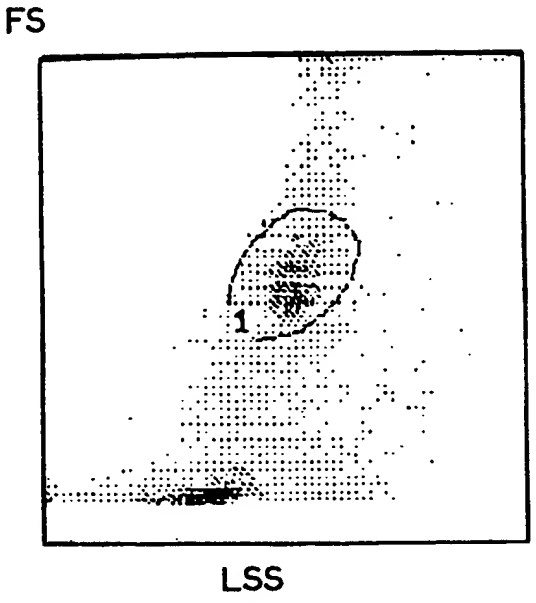


Fig. 2b

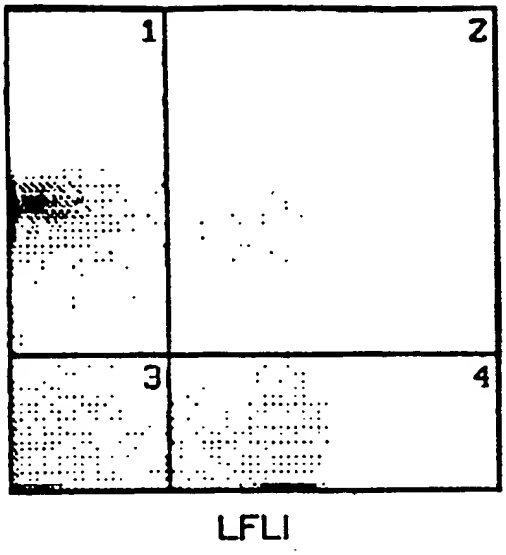


Fig. 2c

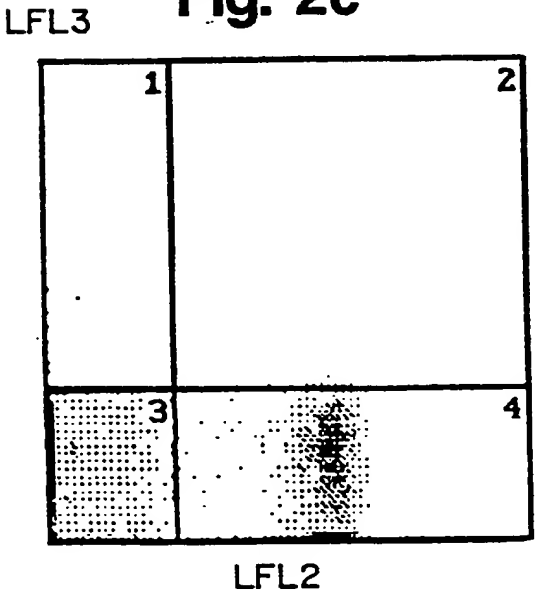
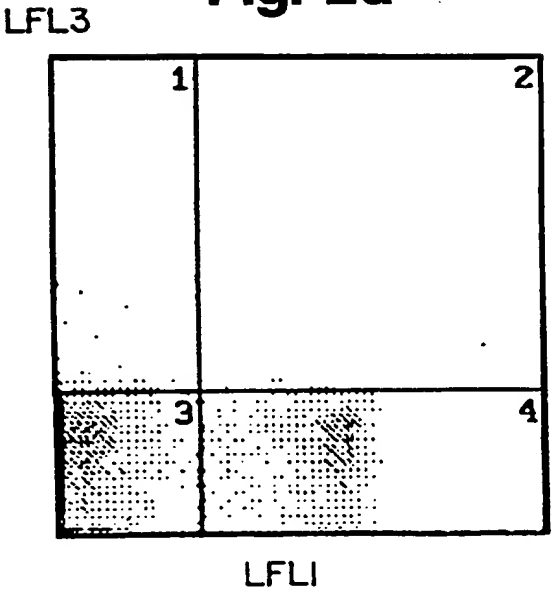


Fig. 2d



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US94/00295

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 21/64, 33/554, 33/533
US CL :435/7.21; 436/172, 176, 519, 546
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.21; 436/172, 176, 519, 546

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,059,518 (KORTRIGHT ET AL) 22 OCTOBER 1991, see column 2, lines 28-46; column 3, lines 25-34; column 10, lines 27-43.	1-11
Y	N.R. ROSE et al, "MANUAL OF CLINICAL LABORATORY IMMUNOLOGY", published 1986 by American Society for Microbiology (Washington, D.C.), see pages 226-235, especially pages 232-234.	1-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 28 February 1994	Date of mailing of the international search report MAR 28 1994
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International ApplicationNo.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CYTOMETRY, Volume 12, issued 1991, G.H. Segal et al, "Concomitant Delineation of Surface Ig, B-Cell Differentiation Antigens, and HLADR on Lymphoid Proliferations Using Three-Color Immunocytometry", pages 350-359, especially pages 352-353.	7,11

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